Rocky Mountain Research Station

A protocol for collecting eDNA samples from snow tracks: filtration (part 2)

Version 1.4, 3 August 2020

Collecting a snow sample from bobcat tracks in Montana.

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Table of Contents

Materials needed:

Provided by NGC:

- *eDNA filtering kits*
- *Peristaltic pump kit and filter stand*
- *Sample storage envelopes*
- *Spare gloves*

User needs to provide:

- *Bleach (concentrated)*
- *Bottled water*
- *Paper towels*
- *Spray bottle (optional)*
- *C-clamp (optional)*

This protocol is designed to be used ***after snow-tracks have been collected and are now ready to be filtered for lab processing.*** *Please refer to "A protocol for collecting eDNA samples from snow tracks: field protocol (part 1)" Version 1.5, June 25, 2020, for an explanation of how to properly collect a snow track containing eDNA.*

Cite as Franklin, Thomas W., McKelvey, Kevin S., Golding, Jessie D., Lex, Justin J., and Schwartz, Michael K. 2020. A protocol for collecting eDNA samples from snow tracks: field protocol (part 1). U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station.

Introduction

The assays used to evaluate environmental DNA (eDNA; e.g., quantitative PCR [qPCR]), are astonishingly sensitive and can detect even a few copies of DNA with high reliability (Wilcox et al., 2013; Dysthe et al., 2018). For example, Jane et al. (2015) placed five small fish in cages and sampled the water at 50 m intervals, with a maximum downstream limit of 240 m. The analysis of eDNA via qPCR detected the caged fish in all 162 eDNA samples. As a result of this sensitivity, eDNA sampling has proved effective for delineating distributions of rare species (Spear et al., 2015; McKelvey et al., 2016;), detecting invasive species (Thomsen et al., 2012; Franklin et al., 2018), and detecting species that are difficult to sample using traditional approaches (Taberlet et al., 2012, Franklin et al., 2019). While it is this extreme sensitivity that enables the collection of eDNA, this same sensitivity makes it vulnerable to contamination. Even a single copy of the target species mitochondrial DNA (far less than is present in a single cell) that erroneously makes its way into a sample will lead to a false positive identification.

While this can seem daunting, we at the National Genomics Center have processed tens of thousands of environmental samples without contamination issues. **The key is to follow the correct steps in both field sample collection, filtration, and laboratory processing.** In the field, you have the advantage of having the world that you are sampling be considered "clean". A tool can be set on the ground without issue, as the ground is part of the environment you are sampling. Only those things that you bring into the environment are considered "dirty": your clothing, the snowmobile, and the outer containers surrounding the sample kits. Field protocols are designed to separate you from the sample (e.g., elbow length gloves) and keep the sample separate from the dirty environment that will transport it back to civilization (e. g. double bagging). Filtering multiple samples in an indoor environment presents different challenges. The room you work in may start "clean"; a hotel room, for example, is unlikely to contain DNA from lynx or coyotes. However, as you bring samples into this space and process them, the space becomes increasingly likely to become "dirty". In a formal DNA laboratory, this problem is controlled by surface sterilizing with bleach or ultraviolet light periodically. However, we are anticipating that you will be working in less formal spaces and are not anticipating periodic sterilization. As such, the protocol relies both on you implementing "clean" processes and isolating of the filtering process from outside contaminators.

The Room

The room should be an area which is clean from DNA of species of interest. This is different from having no DNA; hotel rooms are generally coated with the DNA of many individuals, but we are not looking for human DNA. If, however, a room was previously used by a crew who were part of a catch-and-collar study for Canada lynx, that would be highly problematic. Therefore, a room does not need to be sterile, but the DNA from a wide range of organisms should not be present to reduce potential contamination. When we seek to identify a track in the lab, we may test for multiple species until we have a positive endpoint. For example, let's say that the survey is designed to find wolverines and only putative wolverine tracks are collected. Nevertheless, some of these tracks will

contain no target DNA (e.g. detectable DNA has degraded) and others will have been created by non-target organisms (i. e. track misidentification) and therefore will not contain wolverine DNA. If the wolverine test is negative, we will do other species tests to determine what species made the track: it's important to know whether a track could have been a wolverine track but that the sample contained no DNA or that the track was definitely produced by another species. At a minimum, the room needs to be clean from target DNA sources (scats, hairs, pelts, traps, anything used to handle animals, etc.) and easily cleanable. If the room previously contained materials containing target DNA, the room will need to be sufficiently cleaned with bleach to reduce contamination risks, but we would strongly suggest finding a different room. Please contact us at the NGC if you are not able to provide a clean room for filtering your samples or if you need further instructions on cleaning your filtering room. This is a critical step in providing reliable, quality results.

When you enter the room, you should be as free of target DNA as possible. Wear clothing that has been recently laundered and is separate from what you would wear in the field. Similarly, wear shoes that are different from the shoes you would wear in the field. If you handle wild animals, be particularly aware of the need to choose clothing that is as separate as possible from what you wear handling animals. Develop a protocol that you can maintain and you feel provides adequate separation both from outside sources of DNA and from past use of the room.

Preparing the Room

Prior to filtering samples, the table, counter, desk, etc. should be cleaned with a 10% bleach solution. This can be made by combining 1 part concentrated bleach (purchased from local store) to 9 parts water. If possible, use a spray bottle and paper towels to clean the surfaces. We cannot ship the bleach, so you will be responsible for these sterilization materials. This solution is strong enough to make white spots on clothing and carpets. Wear clothing that you don't care about and if you are forced to use a room with carpeting, cover the carpet with a blue tarp or other waterproof surface to both separate the carpet from the processing and to keep it from getting bleach spots. If you tarp a floor, tape down the edges of the tarp, particularly at doorways and other travel routes both to keep it in place and to prevent tripping.

The Pump and Filtering Kits

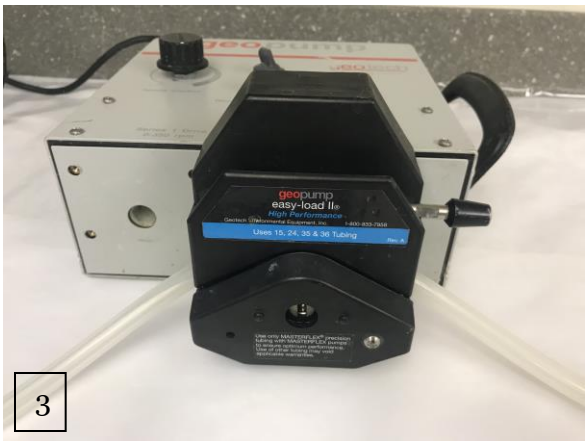
A pump kit consists of a peristaltic pump and hose, battery, outflow bucket, an extra hose adaptor, and a laboratory stand with clamp. We will also provide multiple filtering kits in a white garbage bag, a black garbage bag for trash, and a plastic container with paper envelopes and writing utensils. The filtering kit consists of a filter cup with preloaded filter, nitrile gloves, plastic tweezers, paper towel and a bag of silica (Figure 1).

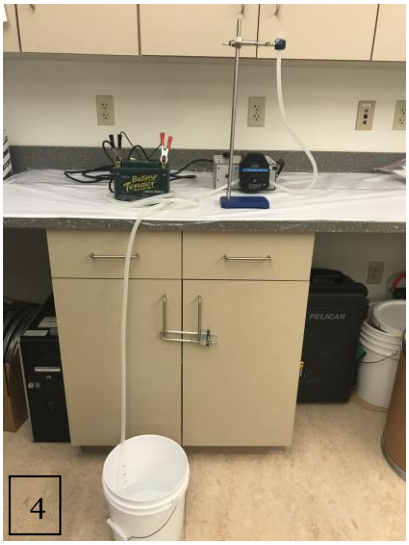
Figure 1. The filtering kit includes gloves, filter cup + pre-loaded filter, tweezers, silica, and paper towel.

Setting Up the Filtration Station

The filtration station needs two separate areas to avoid contamination—one for melting samples, and one for filtering samples.

1. On the filtration table, set the laboratory stand towards the side of the table with the base facing forward.
    a. Optional: It can be helpful to clamp the base of the stand to the table if processing large numbers of samples.
2. Place the clamp on the rod no more than 1 ½" from the top of the rod and secure it tightly (Figure 2).
3. Clamp the base of the plastic adapter (located at the end of the hose) firmly to the stand just below where the adapter flares out (Figure 2).
4. Place the pump on the table behind or next to the stand and thread the hose through the peristaltic pump head (Figure 3).
5. Run the tube down off the table and into drain or bucket (Figure 4).
6. Hook the pump up the battery.
    a. Red on red and black on black
7. Turn the pump and on check for suction at the adaptor with your hand.
    a. If there is no suction, flip the pump direction switch.
    b. Make sure that the speed control dial is on maximum.
8. Turn the pump off. You will only need to do this once. This setup can remain in place if you are filtering multiple samples.
    a. Periodically charging the battery may be necessary if filtering >20 samples.





Figures 2, 3, & 4. These figures demonstrate the proper filtration station setup.

Sample Thawing

The first step is to move snow-track samples out of freezing conditions and into the room. Only move in groups of snow-track samples that you can readily process; **once they are melted, they should be filtered as soon as possible and should not be allowed to sit in a thawed condition**. Once the sample is thawed, the DNA can begin degrading, so find a schedule that works for you to promptly complete the samples at hand. Melt times can vary based on the room temperature and snow volumes, so it can be valuable to thaw a single sample to estimate melt times and become familiar with the protocol.

Sample Filtering

Throughout the instructions below, you will see advice concerning “clean” and “dirty” hands. The critical step in the filtering process where contaminants can get into the filtering system is having a hand at the top of the bag while pouring. It is critical that this hand be clean. The hose, pump, and adaptor are all considered “dirty”. There are many correct ways to do this, but we have provided an example below that we practice.

Ideally, samples will be stored frozen nearby in their white garbage bags. Remove the samples you wish to stage for processing and, keeping them double-bagged, place them on the table not used for filtering; leave the other samples in the freezer or outdoors if the ambient temperature is below freezing. They should be separated so that they thaw at a reliable rate. Once a sample is thawed:

1. Open filter kit. **Without reaching hands into the gallon bag**, remove the paper towel and unfold it onto the table.
2. Pour the gloves, silica desiccant bag, the bagged tweezers and the bagged filter cup out of the gallon bag onto the paper towel. Leave the filter cup and tweezers in their small Ziplocs.
3. Carefully open the silica desiccant bag and place it on the paper towel.
4. With **ungloved** hands, open the outer Ziploc bag of the snow-track sample and pull it wide.
5. Put on one glove and remove the sample bag (Whirl-pac) from the outer Ziploc with the gloved hand and set it on the filtering table. It should stand upright.
    a. The gloved hand is now your dirty hand.
6. Put on the other glove; this hand is your clean hand.
7. Carefully open the small Ziploc containing the filter cup and with it still in the bag, adjust it so the blue end with the stem protrudes from the bag. Grab the hose adapter with your dirty hand and place the cup firmly onto the adapter.
    a. The top of the filter cup should extend above the rod.
8. Using your clean hand, untwist the wires on the sample bag Whirl-pac and spread the wires wide. With your clean hand, make a spout by bending the wires into a “V” shape.
9. Lift off the small Ziploc bag from the filter cup. Using your dirty hand, turn on the pump.

Figures 5 & 6. These figures demonstrate the pouring technique for right handed (figure 5) and left handed (figure 6) filtering. The key here is to never put your “dirty” hand above the filtering up or on the spout.

10. With your clean hand, pinch the bag at the open end of the Whirl-pac opposite of the spout. Hold the base of the Whirl-pac with your dirty hand. Pull the Whirl-pac taut and slowly pour the liquid into the filter cup. See figures 5 & 6.
    a. Be careful not to pour too fast as the filter cup could overflow.
    b. If you get tired, you can set down the Whirl-pac–it should stand upright.
11. Continue slowly pouring until all liquid is gone or the filter clogs.
12. If the filter does clog, refer to "Dealing with Clogged Filters", below. Grasp the filter cup base (blue part) with your dirty hand and remove the top of the cup (clear part) with your clean hand, exposing the filter.
    a. IMPORTANT: When removing the top of the cup, make sure your hand does not go over the cup. Grab the cup from the side.
13. Carefully open the bag containing the tweezers and adjust them so the fixed end sticks out of the bag and you can grab them without touching the pinchers.
14. Using the tweezers, fold the filter in half, then in half again (quarters) and place the folded filter in the silica desiccant filled bag. Seal the bag while removing as much air as possible.
15. Turn the pump off.
16. Use permanent marker to label the silica desiccant bag with the Collection Date, Location Name, Sample ID, Sample "A" or "B", and your initials. Record sampling data from the Whirl-pac onto an envelope, or peel the data sticker from the Whirl-pac and place it on an envelope.
17. Roll up the labeled silica bag with the folded filter, place it into the envelope, and seal the envelope.
18. Place envelope in safe spot away from both the thawing samples and the filtration table.
19. Dispose of all trash (Ziplocs, gloves, paper towels, envelope seal) and place the recyclables (plastic filter cups and bases, tweezers, sharpies) into the black garbage bag.
    b. Be sure to remove the base of the filter cup from the adapter.
    c. All recyclables will be mailed back to the lab for sterilization and reuse.
20. Wipe down table with 10% bleach solution.
21. Repeat steps 1 through 20 until all remaining thawed samples are filtered.
22. Once all thawed samples are filtered, collect a negative filter control (see Negative Filtering Control section below).
23. Wipe down tables and pump kit equipment with a 10% bleach solution.

Negative Filtering Control

When you are finished filtering samples for the day, we request you collect a negative filtering control. The filtering control can be used to check for contamination entered during the filtering process. This consists of following the same protocol as above but instead of a Whirl-pac of snow water, bottle distilled water is used. When filtering, fill the filter cup up ~3 times with distilled water, then continue with step 9. Label as "Negative Filtering Control" and include the date. Note: this negative control is different from the one taken in the field. The field control is taken to check whether contamination is occurring in the field during snow collection. This negative control is taken to check whether contamination is occurring during the filtering process.

Dealing with Clogged Filters

In some cases, you will not be able to filter all the water in a snow sample. If the filter gets very sluggish, slow down the rate at which you are filling the cup. You want the filter to be able to dry itself and not clog completely with water still in the cup. It's a good thing to have only 1 filter per sample, so if you are close to the bottom, spend the extra time and try to get all the water to filter. If, however, you are obviously clogging with a lot of water left, you will need to finish with a second filter. Here the process is identical to the steps above, the only difference being that you mark the bags with filter #1 and #2, then place both into one envelope and note multiple filters were used. Exact volumes per filter are not necessary to record.

Storage & Transportation

Once a sample is filtered and placed in desiccant it is stable and doesn't require freezing or special care in the short term. Keep in a cool, dark, dry space and mail groups of samples to the National Genomics Center within 2 weeks of filtration. If samples cannot be mailed within 2 weeks of filtering, place in a freezer until they can be mailed/transported. When you have finished sampling, mail the pump, stand, clamp, and all the cups and tweezers back to the National Genomics Center.

All samples should be sent to:

Tommy Franklin, eDNA Program Leader
800 East Beckwith Ave.
Missoula, MT 59801
406-542-4171
thomas.franklin@usda.gov

For questions regarding the Multispecies Mesocarnivore Monitoring Program contact:

Jessie Golding, Multispecies Mesocarnivore Monitoring Program Leader
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Missoula, MT 59801
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